# Adult Hippocampal Neural Stem/Progenitor Cells *In Vitro* Are Vulnerable to the Mycotoxin Ochratoxin-A

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The common mycotoxin ochratoxin-A (OTA) accumulates in brain, causes oxidative stress, and elicits a DNA repair response that varies across brain regions and neuronal populations. Neural stem/progenitor cells (NSCs) prepared from hippocampus of adult mouse brain were tested for their vulnerability to the toxin *in vitro*. The following measurements were made in NSC cell cultures in both proliferation and differentiation media: (1) viability (trypan blue exclusion), (2) proliferative activity ([$^3$H]-thymidine uptake), (3) the DNA repair response (oxyguanosine glycosylase activity), and (4) antioxidative response (superoxide dismutase). Cells that had proliferated to 90–100% confluency in the presence of epidermal growth factor and basic fibroblast growth factor were induced to differentiate by removal of the growth factors. OTA, added to the cultures in concentrations of 0.01–100 µg/ml, caused a dose- and time-dependent (6–72 h) decrease in viability of both proliferating and differentiating NSC. Proliferating NSC exhibited a greater vulnerability to the toxin than differentiated neurons despite robust DNA repair and antioxidative responses. Preconditioning of NSC with a 12-h incubation with the pro-oxidant diethyl maleate increased DNA repair activity and, subsequently, provided a moderate degree of neuroprotection. Overall, these results lead to speculation that OTA exposure may contribute to impaired hippocampal neurogenesis *in vivo*, resulting in depression and memory deficits, conditions reported to be linked to mycotoxin exposure in humans.

*Key Words:* neural stem/progenitor cells; hippocampus; ochratoxin-A; mycotoxin; oxidative stress; DNA repair; 8-oxoguanine glycosylase-1 (OGG1); superoxide dismutase (SOD); diethyl maleate (DEM).

The adult brain retains a reservoir of stem-progenitor cells in the hippocampal ''neurogenic zone'' capable of proliferative activity throughout life (Palmer *et al.*, 1997). Regulation of neurogenesis in hippocampus (HP) is poorly understood, but it is known that injury, irradiation, drugs, and endogenous factors such as hormones and trophic factors impact neurogenesis (Campbell and Macqueen, 2004; Chen *et al.*, 2006; Grote *et al.*,

2005; Kawai *et al.*, 2006; Lagace *et al.*, 2006; Nacher and McEwen, 2006; Rossi *et al.*, 2006). Cranial irradiation has been reported to produce a deficit in neurogenesis that occurs many months after the insult, and the inability to produce new neurons in HP may be associated with cognitive deficits observed in patients who undergo therapeutic cranial irradiation (Byrne, 2005; Monje and Palmer, 2003; Monje *et al.*, 2002).

There are likely to be many unrecognized environmental agents capable of inhibiting hippocampal neurogenesis. Ochratoxin-A (OTA), one of a group of toxic fungal metabolities (mycotoxins) found in *Aspergillus* and *Penicillium* genera has been shown to affect proliferation and migration of neurons in the developing rodent brain (Fukui *et al.*, 1992) and, therefore, may also impact neurogenesis in adult HP. OTA is a natural contaminant present in feed and food and has been found in all parts of the world in the blood of animals and humans after consumption of contaminated food (Abarca *et al.*, 1994; Bayman *et al.*, 2002). Even individuals with no obvious source of exposure have detectable levels of OTA in their blood (Jonsyn-Ellis, 2000; Kuiper-Goodman and Scott, 1989).

The developing brain is highly susceptible to the deleterious effects of OTA (Hayes *et al.*, 1974, 1977; Wangikar *et al.*, 2004). DNA synthesis associated with cellular proliferative activity during development is impaired by the toxin and results in microcephaly in rodents (rats and mice) exposed *in utero* (Fukui *et al.*, 1992). Mice (3 months old), offspring of pregnant mice treated with a single dose of OTA at 11 days gestation, exhibited DNA content reduction to 80% of control in cerebral hemispheres (cerebral cortex and subjacent white matter, HP, and amygdala) and to 90% of control in remainder of the brain (basal ganglia, diencephalon, and mesencephalon) (Tamaru *et al.*, 1988). In cell culture studies, OTA has been shown to inhibit both proliferation and differentiation of fetal midbrain (MB) cell cultures (Hong *et al.*, 2000). OTA significantly reduced protein and DNA levels as well as [$^3$H]-thymidine incorporation into DNA in both embryonic MB and in limb bud cells in a similar concentration-dependent manner (Hong *et al.*, 2000).

The present study was designed to test the hypothesis that adult neural stem/progenitor cells (NSC) are especially vulnerable

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to the common mycotoxin OTA. Based on OTA's capacity to produce oxidative DNA damage across brain regions *in vivo* (Sava *et al.*, 2006a), a major determinant of cellular vulnerability to the mycotoxin was hypothesized to be the ability to repair oxidative damage to DNA. Earlier studies demonstrated that proliferating PC12 cells in the presence of nerve growth factor (NGF) were less vulnerable than differentiated cells (absence of NGF) to the pesticide dieldrin because proliferating cells exhibited greater capacity to repair DNA (Stedeford *et al.*, 2001). Therefore, it was postulated that the DNA repair response elicited by the toxin would impact on the capacity of NSC to proliferate and/or develop into mature neurons in the adult HP.

To test the extent and the mechanism for which OTA is toxic for NSC *in vitro*, the effects of OTA were systematically investigated in cell cultures of proliferating and differentiating NSC isolated from HP of adult mice (2–3 months old). The following variables were measured in cell cultures of NSC in both proliferation and differentiation media: (1) viability, (2) proliferative activity, (3) the DNA repair response, and (4) antioxidative response. Finally, studies were performed in preconditioned NSC that exhibited enhanced 8-oxoguanine glycosylase-1 (OGG1) activity to determine the extent to which DNA repair activity was a critical determinant of vulnerability to the toxin in these cells. NSC were preconditioned with the pro-oxidant diethyl maleate (DEM), a compound that decreases cellular glutathione levels (Esteban-Pretel and Pilar Lopez-Garcia, 2006). DEM was previously shown to increase DNA repair activity (OGG1) in a brain region–specific manner (Cardozo-Pelaez *et al.*, 2002). The increased repair activity correlated with a reduction in the levels of oxidative DNA damage. The brain region–specific capacity to deal with increased oxidative damage to DNA was hypothesized to be responsible, in part, for the vulnerability of specific neuronal populations to aging, toxicants, and neurodegenerative diseases.

The potential clinical relevance of this study can be found in reports that human mycotoxin exposure (but not specifically OTA) is significantly associated with memory loss, learning and attention deficits, processing speed, and debilitated executive functions (Gordon and Cantor, 2004; Gordon *et al.*, 2004; Rea *et al.*, 2003).

## MATERIALS AND METHODS

***Preparation of NSC in monolayers from HP of adult mice.*** NSC cells were prepared from HP of adult brains of 2- to 3-month-old C57BL/6J mice using a modification of a previously described method (Scheffler *et al.*, 2005). Hippocampi were dissected under a stereomicroscope and transferred to a 35-mm plate containing PBS plus 0.5% bovine serum albumin. Sterile scalpel-minced tissue was then removed to a fresh 14-ml tube where the tissue pieces were allowed to settle to the bottom. After discarding the supernatant, 5 ml of a solution of 0.05% trypsin/EDTA was added. The tissue was triturated with a fire-polished pipette followed by incubation of the cell suspension at 37°C for 10 min or until the clumps of tissue were digested. The cells were removed from the incubator, and DNAse (1:10 dilution) was added, followed by

trituration with a fire-polished pipette to obtain a single-cell suspension. An equal volume of Dulbecco's modified Eagle's medium (DMEM)/F12 plus 10% fetal bovine serum (FBS) was added to stop the activity of trypsin, followed by centrifugation of the cell suspension for 5 min at 1200 rpm. The pellet was resuspended in 5 ml of DMEM/F12 + 10% FBS. Additional DNAse (1:10 dilution) was added to obtain a single-cell suspension by another round of trituration. The washes and DNAse treatment were repeated (1–2 more times) until a homogeneous single-cell suspension without cell clumps was obtained. The final pellet was resuspended in DMEM/F12, the final volume was determined, and viable cell count was performed. These cells were then seeded in T75 flasks and incubated for 3 days followed by collection of the media, centrifugation, and resuspension of the cell pellet to reseed the flask. The culture media was changed every week until cell colonies appeared. Cell colonies were collected, dissociated, and plated in 96 wells at a density of 1 cell/well in proliferation media (DMEM/F12 plus 2% FBS, epidermal growth factor [EGF], and basic fibroblast growth factor [bFGF] 20 ng/ml). The cultures plates were placed on a level tray in a 37°C humidified incubator (5% $CO_2$). Culture media was changed once per week until the cells reached confluence. Cells were then either cryopreserved or transferred to multiwell plates for studies of the effects of OTA.

***Chemicals.*** OTA (7-carboxyl-5-chloro-8-hydroxyl-3,4-dihydro-3-*R*-methylcoumarin-7-L-b-phenylalanine) was purchased from Sigma Co. (St Louis, MO, 99.0% purity). Stock solutions of OTA (1 mg/ml) were dissolved in the vehicle dimethyl sulfoxide. OTA was then diluted in cell culture N5 medium to achieve final concentrations ranging from 0.01 to 100 μg/ml. DEM was purchased from Sigma Co. and diluted in cell culture N5 medium to achieve the final concentrations ranging from 0.1 to 1mM.

***Studies of toxicity in proliferating and differentiating NSC.*** Cells from passage 2 to 6 (P2–6) were plated in either 35-mm multiwell plates for biochemical assays or multiwell culture slides containing proliferation media (DMEM/F12, 2% FBS, EGF, and bFGF 20 ng/ml). To induce differentiation, the cells from passage 2 to 6 (P2–6) were plated on glass coverslips or multiwell slides coated with 10 μg/ml poly-L-ornithine (Sigma Co.) at densities of 2 × $10^5$ cells per cm$^2$. The cells were proliferated to 90–100% confluency and were induced to differentiate by removing the growth factors from the culture media (DMEM/F12 and 2% FBS) (Mignone *et al.*, 2004). OTA was added to the cultures in a range of concentrations from 0.01 to 100 μg/ml. In preconditoning studies, DEM (0.1–1mM) was added to NSC in the differentiation medium for 12 h, followed by removal of the medium and replacement with fresh differentiation medium for 72 h followed by assays of viability and DNA repair.

***Analysis of viability.*** The trypan blue exclusion assay was used to determine cell viability of attached cells. Cells were gently raised from the culture dishes by brief incubation with 0.05% trypsin, followed by addition of culture medium with 10% FBS to end the action of trypsin. A cell suspension was prepared containing ~$10^6$ cells/ml and diluted 1:1 using a 0.4% trypan blue solution. The counting chambers of a hemocytometer were loaded with the dilution. After 1–2 min, the number of stained cells and total number of cells were counted. The calculated percentage of unstained cells represented the percentage of viable cells.

***[$^3$H]-thymidine incorporation by NSC.*** Cells were plated as described above and incubated with OTA at time zero. At various intervals after OTA (0, 24, 48, and 72 h), 37,000 Bq/ml (500 pmol/m;) [$^3$H]-thymidine (PerkinElmer Life and Analytical Sciences, Inc., MA) was added to the cultures for 4 h, then cells that adhered to the plate were harvested, and [$^3$H]-thymidine incorporated into DNA was determined. The specific activity of [$^3$H]-thymidine was 74 Bq/pmol. Uptake was normalized and expressed as pmol/hour/million cells.

***Analysis of DNA repair.*** Enzymatic activities of OGG1 were measured according to a protocol established in our laboratory (Cardozo-Pelaez *et al.*, 2000). Briefly, cells were sonicated in homogenization buffer, spun at 14,000 rpm for 30 min, and supernatants were collected. The OGG1 activities in supernatants were determined using $^{32}$P-labeled duplex oligonucleotide containing 8-oxodG as incision substrate. Products of the reaction were separated

on denaturing 20% polyacrylamide gel and detected with Biorad-363 Phosphoimager System. The activity of OGG1 was calculated as the amount of radioactivity in the band representing specific cleavage of the labeled oligonucleotide over the total radioactivity. Data were normalized to equal concentration of protein, the concentration of which was measured using the bicinchoninic acid assay (Smith *et al.*, 1985).

*Superoxide dismutase assay.* Determination of superoxide dismutase (SOD) activity in the cell cultures was based on inhibition of nitrite formation in reaction of oxidation of hydroxylammonium with superoxide anion radical (Elstner and Heupel, 1976). Nitrite formation was generated in a mixture containing 25 μl xanthine (15mM), 25 μl hydroxylammonium chloride (10mM), 250 μl phosphate buffer (65mM, pH 7.8), 90 μl distilled water, and 100 μl xanthine oxidase (0.1 U/ml) was used as a starter of the reaction. The inhibitory effect of inherent SOD was assayed at 25°C during 20 min of incubation with 10 μl of brain tissue extracts. Determination of the resultant nitrite was performed upon the reaction (20 min at room temperature) with 0.5 ml sulfanilic acid (3.3 mg/ml) and 0.5 ml α-naphthylamine (1 mg/ml). Optical absorbance at 530 nm was measured on Ultrospec III spectrophotometer (Pharmacia LKB) to evaluate the total concentration of SOD. The concentration of cytosolic SOD was determined in parallel reactions supplemented with 10mM KCN to inhibit mitochondrial SOD. The concentration of mitochondrial SOD was calculated as the difference between amount of total and cytosolic fractions of SOD. The results were expressed as units of SOD activity calculated per milligram of protein. The amount of protein in the samples was determined using the bicinchoninic acid (Smith *et al.*, 1985).

*Immunocytochemistry.* NSC cultures that were induced to differentiate into neurons, and glia were stained and visualized using immunocytochemical methods. The primary antibodies included: nestin (Pharmingen) as marker of early neural progenitors; class III β-tubulin or TuJ1 (Sigma) to identify neurons at an early stage of development; glial fibrillary acidic protein (GFAP; Sternberger Monoclonals) to identify astrocytes; and GALC (Sigma) to identify oligodendrocytes. The cultures were then washed in PBS before being placed in secondary antibody conjugated to fluorescein for 2 h. The cultures were rinsed in PBS, mounted, and coverslipped. Examination of cells employed an Olympus fluorescence microscope with appropriate filters. Digital images were captured at ×100 and ×200 magnification (×10 and ×20 objectives) with an Optronics digital camera.

*Statistical analysis.* Analysis of data on viability and biochemical responses to the mycotoxins as a function of time and concentration of OTA employed two-way ANOVA followed by *t*-tests appropriately corrected for multiple comparisons. Linear regression analyses were carried out for correlation studies. Statistical tests were performed with Graphpad statistical software (Sorrento, CA).

## RESULTS

### OTA Is Toxic for (NSC) In Vitro

NSC were prepared from HP of 2- to 3-month-old C57BL.6J male mice. After plating cells in proliferation and differentiation conditions, the effects of OTA on viability was determined using the trypan blue exclusion method. Viability of cells decreased as a function of OTA concentration and duration of exposure (Fig. 1). Interestingly, cells undergoing active proliferation were more susceptible to OTA than when they were in differentiation media. Viability decreased to 41% by 72 h in the proliferation media compared to a decrease in viability to 62% in the differentiation media. Also of interest is the fact that the proportion of viable cells decreased with time when cells were undergoing differentiation, even in the absence of the toxin indicated by C in Fig. 1). To measure the effects of OTA on proliferation, the hippocampal NSC were pulse-labeled with [3H]-thymidine 4 h prior to harvesting cells at various intervals after incubation with OTA (Fig. 2). Under control conditions, the number of cells increased to reach a plateau by 48 h, and this was associated with a decrease in rate [3H]-thymidine uptake. OTA caused a concentration- and time-dependent decrease in [3H]-thymidine uptake, indicating a significant inhibition of DNA synthesis. This corresponded to a diminished rate of cellular proliferation as shown by the decreased number of cells that accumulate over time following incubation with 10 and 100 μg/ml of OTA (Fig. 2, right panel).

The effect of OTA in differentiating NSC was qualitatively assessed by immunocytochemical analysis. The cells were plated as single-cell suspensions in polyornithine-coated
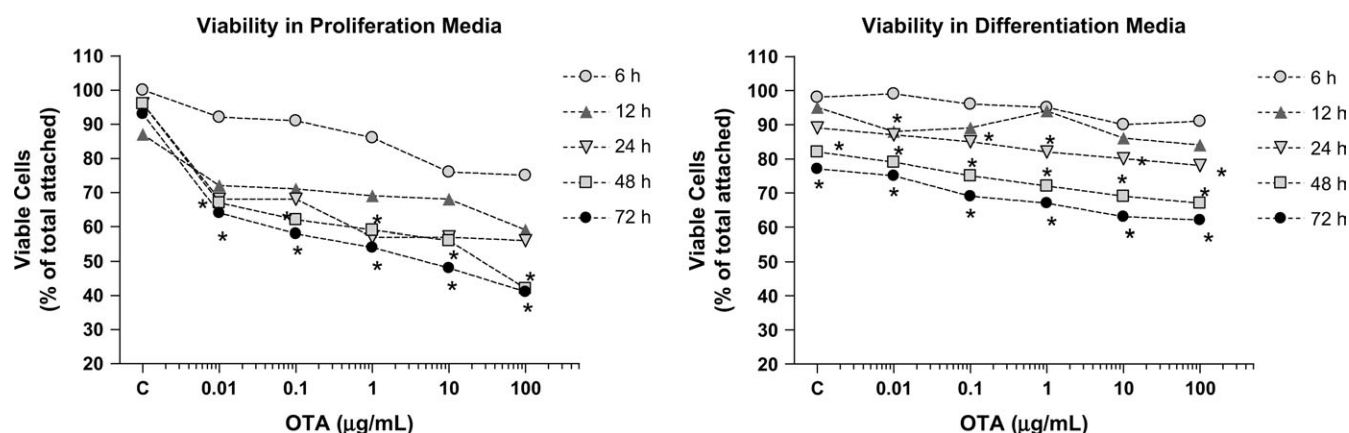
**FIG. 1.** Effects of OTA (0.01–100 μg/ml) on hippocampal neural progenitors from adult mouse brain. Cells were plated in proliferation medium (left panel) or differentiation medium (right panel). OTA was added to the media, and viability of cells was determined (number of cells that extruded trypan blue/total number of cells × 100). C, control condition in which no toxin was added. SEM (not shown) was less than 12% of the means at all points. Two-way ANOVA showed that concentration and time each accounted for significant percentages of total variance ($p < 0.0001$) in viability of proliferating cells (left panel) and differentiating cells (right panel). Statistically significant differences ($p < 0.01$) from corresponding points at 6 h are denoted by asterisks (*t*-tests with Bonferroni correction).
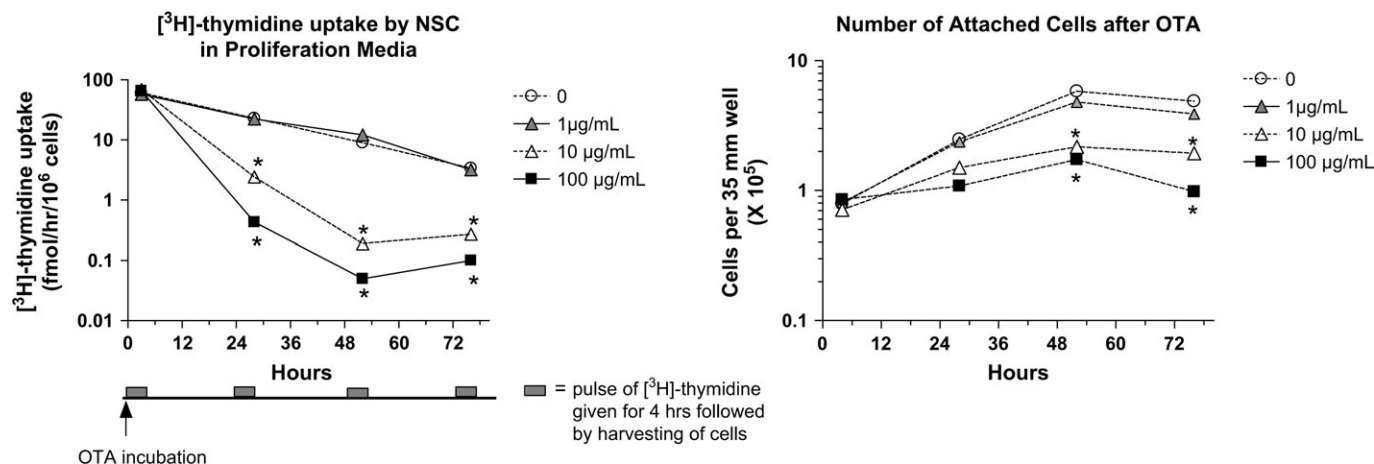
**FIG. 2.** Effects of OTA on rate of DNA synthesis and cellular proliferation. Left panel: Effects of OTA on rate of [³H]-thymidine uptake by adult hippocampal NSC in proliferation media. A pulse of [³H]-thymidine was added to the medium for 4 h before harvesting attached cells at each time point. OTA caused a concentration- and time-dependent decrease in [³H]-thymidine uptake, normalized to number of cells per culture well and expressed as fmol/h/10⁶ cells. Right panel: Time course of effects of OTA on number of attached cells. OTA inhibited proliferation of cells at concentrations of 10 and 100 µg/ml. Asterisks indicate significant difference from control condition, using *t*-tests with corrections for multiple comparisons ($p < 0.05$).

4-well culture slides containing N5 medium containing 2% FBS, 20 ng/ml EGF, and 20 ng/ml bFGF for 4 days. After changing the media (removing growth factors EGF and bFGF), OTA was then added to the medium for 72 h. Cultures were then processed (on day 10) for immunocytochemical detection of TuJ1, GFAP, and GALC (see Fig. 3.) The number of surviving cells, regardless of phenotype, was significantly diminished. In addition, the surviving cells exhibited morphological changes, with shrinkage of cell bodies and loss of neural fibers.

*OTA Impacts Oxidative DNA Repair (OGG1 activity)*

Along with these changes in viability and rate of proliferation, OTA elicited an increase in repair of oxidative DNA damage (OGG1 activity) of the NSC in a dose- and time-dependent manner (Fig. 4). While in the proliferative phase, maximal OGG1 activity of the NSC attained 30 fmol/h/mg at the highest concentrations of OTA. In the differentiation media, maximal OGG1 activity of the NSC never exceeded 15 fmol/h/mg. It is important to note that the baseline levels of DNA repair (OGG1), prior to addition of the toxin, were significantly elevated in the proliferating cells compared to those in differentiating cells (notice different scales of y-axis in lower panels of Fig. 4). In addition, the time course of OGG1 activities in the absence of toxin was significantly different in proliferating and differentiating cells (Fig. 5). The OGG1 activity in differentiating cells was 5 fmol/h/mg at 6 h and gradually increased to reach levels of 12.3 fmol/h/mg at 72 h. In contrast, proliferating cells exhibited levels of 13.9 fmol/h/mg at 6 h and remained stably elevated up to 72 h. Along with these baseline differences, there was a trend to decreased viability in differentiating cells, but this did not reach statistical significance.

When the relationship between OGG1 activity and rate of [³H]-thymidine uptake was analyzed, OGG1 was found to be inversely related to uptake of the radionucleotide (Fig. 6). This is of interest since the DNA repair process is known to involve uptake of nucleotides to some extent, but it is negligible compared to the rate of [³H]-thymidine uptake in proliferating cells.

OTA also elicited an increase in total, cytoplasmic, and mitochondrial SOD activities (Fig. 7). Similar to OGG1 activities, SOD activities were higher at baseline in proliferating cells than in differentiating cells. Together with the augmented DNA repair activities, the increased SOD activities reflect a concerted response to oxidative stress elicited by OTA.

*Preconditioning with a Pro-oxidant that Upregulates OGG1 Is Neuroprotective*

To test whether preconditioning with a low dose of a pro-oxidant that mildly stimulates baseline OGG1 would modify cell vulnerability, DEM at a concentration of 100µM was added to NSC in differentiation media for 12 h before replacing the medium with fresh medium containing OTA for 72 h. Earlier work with DEM *in vivo* showed that a small dose could elicit a transient upregulation of OGG1 and a decrease in oxidative DNA damage in mouse brain regions (Cardozo-Pelaez *et al.*, 2002). In the present study, treatment of NSC with 100µM DEM alone caused a 2–4% decrease in viability and nearly a 100% increase in OGG1 activity to ~16 fmol/h/mg protein in the differentiating NSC (Fig. 8). Pretreatment of cultures with DEM (100µM) 12 h before adding OTA significantly increased viability of NSC cells when assessed after 72 h of incubation with OTA (Fig. 9). Interestingly, OGG1 activity was decreased in the DEM-preconditoned cultures treated for 72 with the entire range of concentrations of OTA.
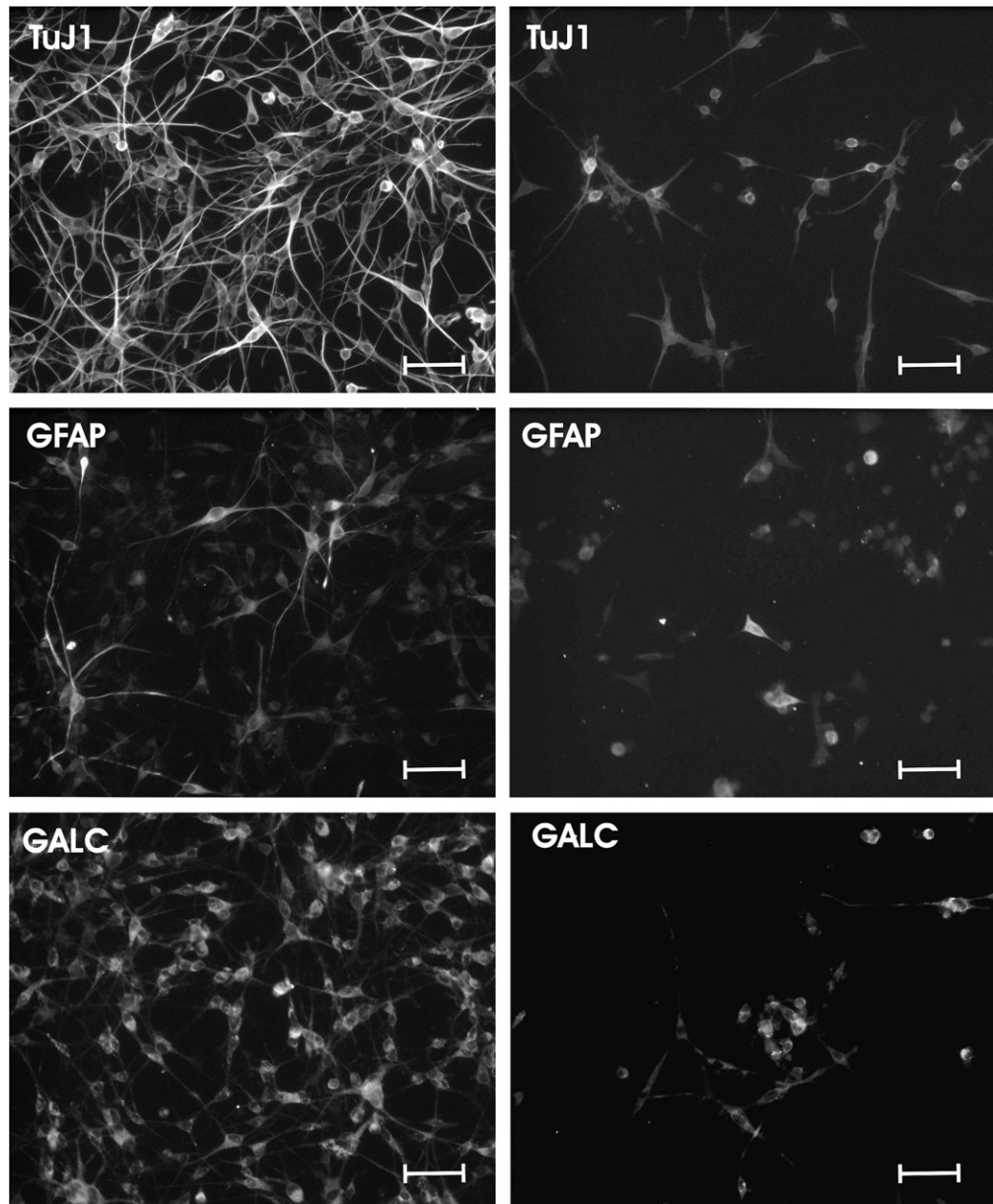
**FIG. 3.** The number and morphology of all three neural phenotypes, neurons, astrocytes, and oligodendrocytes was affected by OTA (10 μg/ml over 72 h added after 7 days of differentiation). The column on the left shows the cells in the control condition (differentiation medium for 10 days) and the column on the right is the OTA-treated condition (differentiation medium for 10 days). TuJ1+ cells = young neurons; GFAP+ cells = astrocytes; GALC+ cells = oligodendroglia. Scale marker = 50 μm

## DISCUSSION

The contribution of OTA to human and animal diseases has been previously reviewed (Marquardt and Frohlich, 1992). OTA has been suspected to play a role in the etiology of Balkan endemic nephropathy and has been reported to have immuno-toxic, genotoxic, and carcinogenic effects (Creppy *et al.*, 1985; Huff, 1991; Pfohl-Leszkowicz *et al.*, 1993a,b; Zepnik *et al.*, 2001). OTA has been reported to be directly neurotoxic at doses below threshold for renal toxicity (Belmadani *et al.*, 1998; Sava

*et al.*, 2006a,b). Moreover, the developing nervous system appears to be especially vulnerable to the toxin (Fukui *et al.*, 1992).

Assays on human blood from several countries have reported concentrations of OTA as high as 40 ng/ml (Kuiper-Goodman and Scott, 1989). Concentrations of OTA studied *in vitro* in the present study ranged from 0.01 to 100 μg/ml. The lower concentrations used *in vitro*, 0.1 μg/ml (100 ng/ml), were comparable to brain levels (94 ng/g of brain tissue) attained in rats that were gavaged with 289 μg/kg body weight every 48 h for
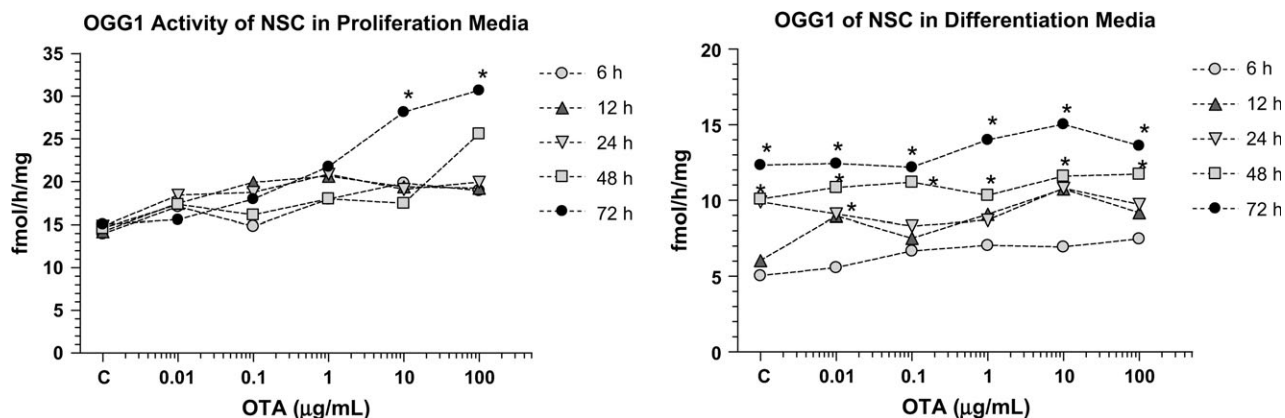
**FIG. 4.** Effects of OTA (0.01–100 μg/ml) on OGG1 activity in hippocampal neural progenitors from adult mouse brain. Cells were plated in proliferation medium (left) or differentiation mediim (right). OGG1 activity was also determined in NSC under both conditions SEM (not shown) was less than 10% of the means in the OGG1 determinations. Two-way ANOVA showed that concentration, but not time, accounted for a statistically significant percentage of total variance ($p < 0.0001$) of OGG1 in proliferating cells (left panel). Both time and concentration contributed to total percentage of variance of OGG1 activity in differentiation media ($p < 0.001$). Statistically significant differences ($p < 0.01$) from corresponding points at 6 h are denoted by asterisks (t-tests with Bonferroni correction).

6 weeks (Belmadani *et al.*, 1998). These rat doses are well beyond the estimated tolerable daily intake in humans, which ranges from 0.2 to 4.2 ng/kg body weight (Kuiper-Goodman and Scott, 1989). Nevertheless, until the study of the effects of OTA on adult hippocampal neurogensis is conducted *in vivo*, it will not be possible to assess the clinical relevance to humans of environmental OTA exposure.

Until now, there was no information in the literature related to the effects of mycotoxins on adult hippocampal NSC which normally retain mitotic competence and capacity to generate new neurons throughout life (Palmer *et al.*, 1997; Song *et al.*, 2002). The function of these ever changing new populations of hippocampal neurons is unknown but may relate to formation of temporal associations in episodic memory (Aimone *et al.*, 2006). The present *in vitro* study demonstrated that OTA decreased viability of adult NSC in a dose- and time-dependent manner. Cells in both proliferation and differentiation media

were vulnerable, but sensitivity to the mycotoxin was greater in the proliferating cells. Along with decreased viability, OTA elicited a pronounced oxidative stress evidenced by a robust increase in total and mitochondrial SOD activity. The toxin also significantly increased OGG1 activity, the DNA repair enzyme responsible for initiating base excision repair of oxidative damage to DNA. The DNA repair response appears to be an important determinant of cellular vulnerability to the toxin, evidenced by the observation that preconditioning with an agent that upregulated OGG1 activity increased viability of cells compared to cultures exposed only to the toxin. Curiously, the DEM-preconditioned cultures exposed to OTA ultimately exhibited a lower OGG1 activity than did the cultures exposed to OTA alone. This phenomenon may be similar to ischemic preconditioning in which exposure of myocardial or brain tissue to brief, repeated periods of vascular occlusion renders the myocardium or brain tissue resistant to the deleterious
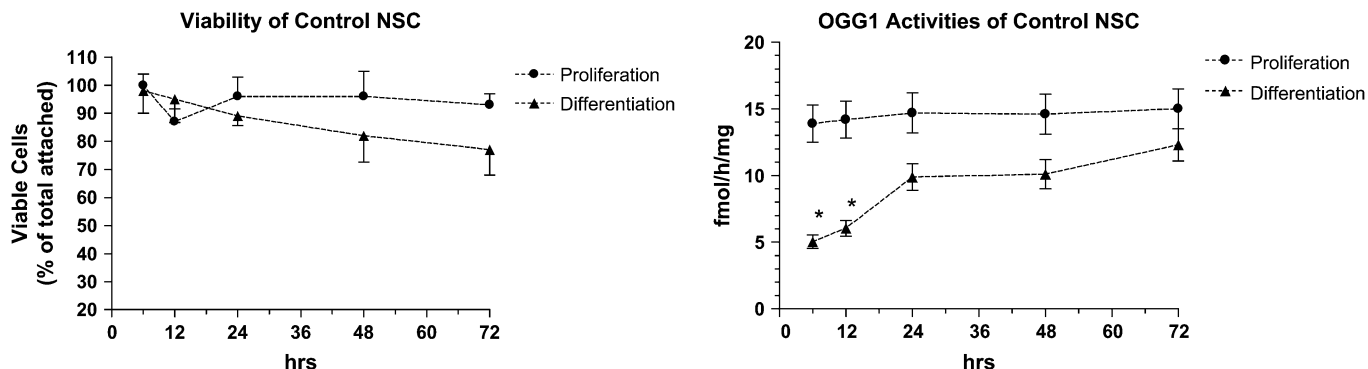


**FIG. 5.** Viability and OGG1 activities of NSC in proliferation and differentiation media under control conditions. Left panel: The time course of viability in proliferating and differentiating NSC are not significantly different (two-way ANOVA, $p = 0.22$). Right panel: Time course of OGG1 activities in proliferating and differentiating NSC. Two-way ANOVA of OGG1 activity reveals that condition (proliferation vs. differentiation) contributed 70% of total variance ($p = 0.007$). *Post hoc* comparison of values at each time point shows statistically significant difference ($p < 0.01$) at 6 and 12 h (t-tests with Bonferroni correction for multiple comparisons).
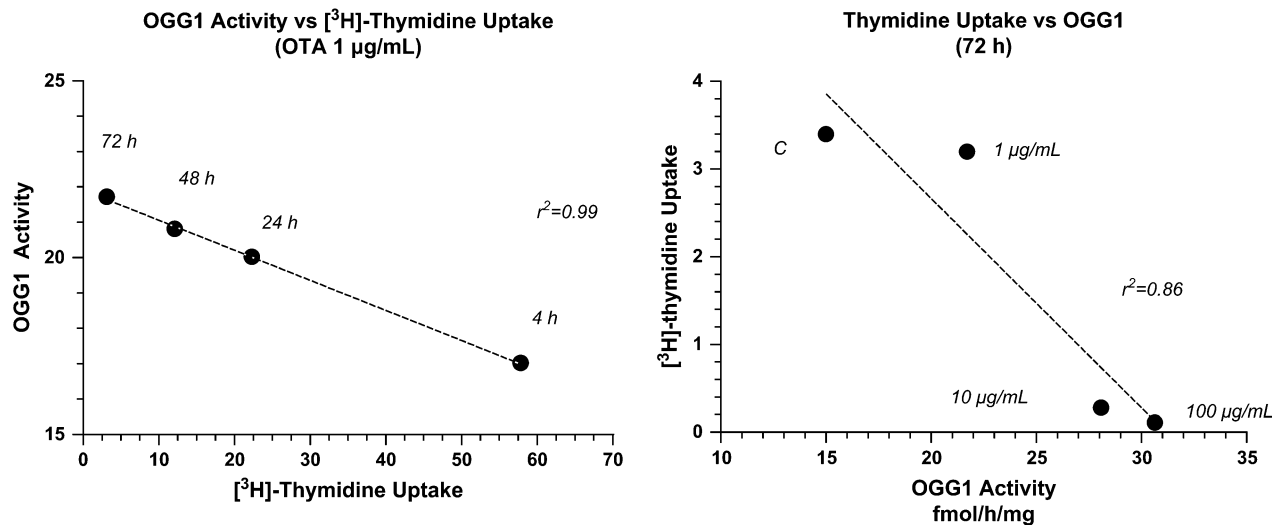
**FIG. 6.** Left panel: Inverse relationship between OGG1 activity and [³H]-thymidine uptake in proliferating cells incubated with OTA (1 µg/ml) as a function of time. Linear regression analysis reveals slope is significantly different from zero and correlation coefficient = 0.99. Right panel: Relationship between OGG1 and [³H]-thymidine uptake as function of dose of OTA at 72 h. Correlation coefficient = 0.86.
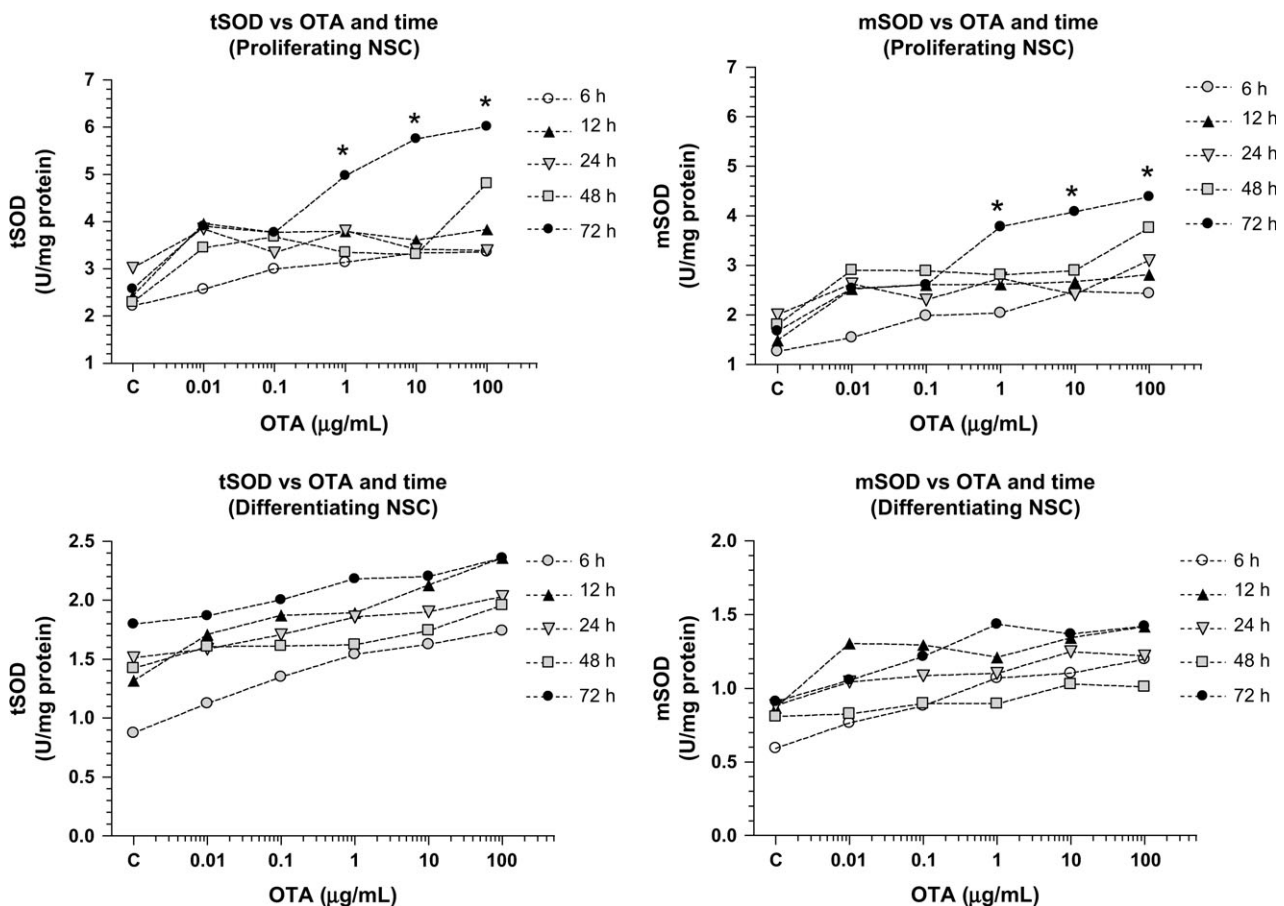


**FIG. 7.** Upper panels: Effects of OTA on total SOD activity (tSOD) and mitochondrial SOD (mSOD) in proliferating cells. Lower panels: Effects of OTA on tSOD and mSOD in differentiation media. Each point is the mean value determined from analysis of six separate culture wells for each concentration of OTA. The SEM is not indicated but was ≤ 10% for each point. Two-way ANOVA revealed that both time and concentration contributed significantly to total variance ($p < 0.001$) in both the proliferating and differentiating cells. Asterisks indicate significance difference ($p < 0.05$) between curves at 72 and 6 h ($t$-tests with correction for multiple comparisons).
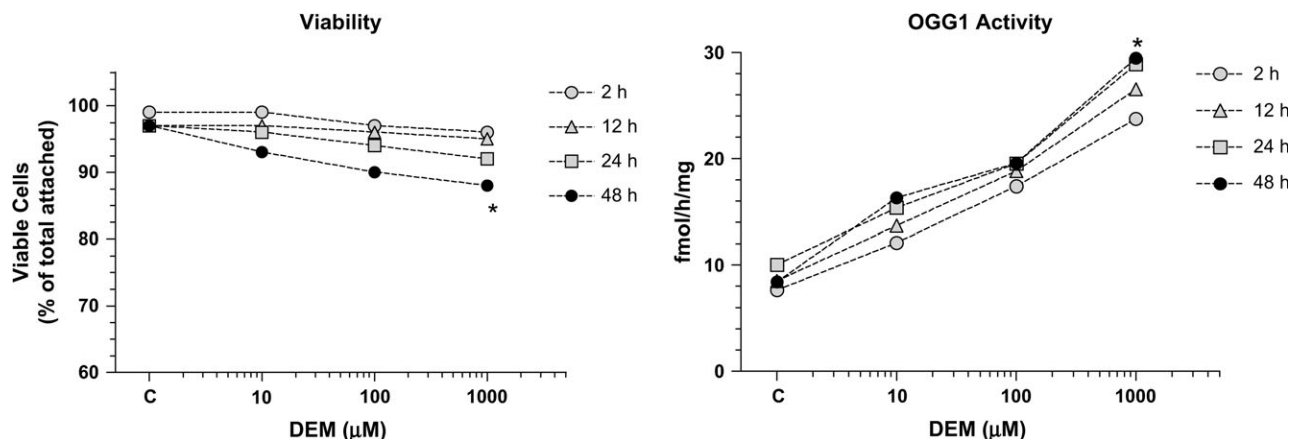
**FIG. 8.** Effects of the pro-oxidant DEM on viability (left panel) and OGG1 activity (right panel) of NSC. Two-way ANOVA shows that both concentration and time contribute significantly to total variance in viability and in OGG1 activity ($p < 0.003$). The $T$-tests with Bonferroni correction for multiple comparisons were performed. Asterisk indicates statistically significant differences ($p < 0.01$) in viability and OGG1 activity between the value at 48 h and corresponding value at 2 h.

effects of prolonged episodes of ischemia or reperfusion (Murry *et al.*, 1986). The mechanism responsible for protective effects of ischemic preconditioning is complex, not completely understood and is beyond the scope of this report. However, reactive oxygen species play a crucial role in signal transduction mediated by preconditioning. For example, pretreatment with a hydroxyl radical scavenger was paradoxically shown to prevent the protective effect of ischemic preconditioning in cardiac tissue (Das *et al.*, 1999; Skyschally *et al.*, 2003). In the present report, pretreatment with a pro-oxidant agent paradoxically protected against the actions of a major pro-oxidant, the myoctoxin OTA.

OTA is well known to create oxidative stress. The mycotoxin inhibits succinate-dependent electron transfer activities of the mitochondrial respiratory chain in hepatic tissues, and at higher concentrations, OTA also inhibits electron transport at complex

I (Aleo *et al.*, 1991; Wei *et al.*, 1985). A consequence of impeding mitochondrial electron transport with a complex 1 inhibitor is the generation of oxygen-free radicals that damage macromolecules (Hasegawa *et al.*, 1990). OTA administered at doses below the renal toxicity threshold has been reported to increase oxidative stress and cause oxidative DNA damage in mouse brain tissue (Sava *et al.*, 2006a,b). A single dose of OTA elicited a dose- and time-dependent increase in oxidative DNA damage, as measured with the "comet assay," across all brain regions up to 72 h, with peak effects noted at 24 h in MB, caudate/putamen, and HP (Sava *et al.*, 2006a). Oxidative DNA repair activity (OGG1) was biphasic with an initial depression in all regions at 6 h followed by improvement to control levels in cerebellum but not in other regions of brain. In contrast to OGG1, other indices of oxidative stress (lipid peroxidation and SOD activity) exhibited a monophasic increase over time
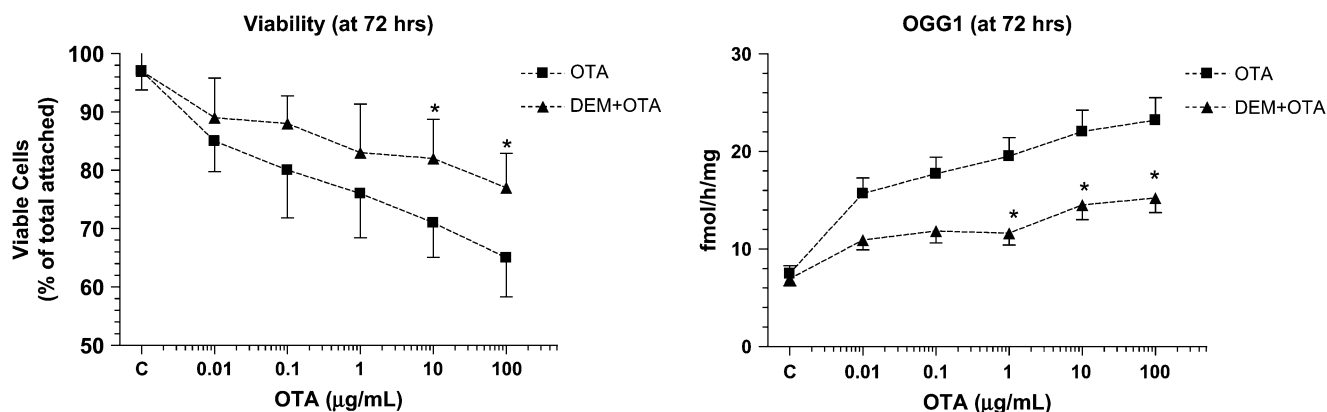


**FIG. 9.** Effects of preconditioning with a pro-oxidant on OTA toxicity. Left panel shows effects of DEM pretreatment on NSC viability. DEM ($100 \mu M$) was added to the cultures for 12 h followed by replacement with medium containing OTA ($0.01–100 \mu g/ml$) for 72 h. Right panel indicates OGG1 activity in a parallel set of cultures. Viability of NSC was improved with DEM pretreatment (left panel). Corresponding to the increased viability provided by DEM pretreatment, mean OGG1 activities were decreased to less than 15 fmol/h/mg (right panel). Two-way ANOVA showed that both OTA concentration and preconditioning contributed significantly to variance. Asterisks indicate statistically significant differences ($p < 0.05$) between treatments ($t$-tests with Bonferroni correction).

throughout the brain (Sava *et al.*, 2006a,b). These results suggested that variations in the DNA repair response were an important determinant of vulnerability to the toxin in different regions of brain.

The present findings that proliferating NSC appear to be more vulnerable to OTA than differentiating cells suggests that its mechanism of action as an inhibitor of mitochondrial oxidative metabolism may be less critical than its interference with DNA synthesis and mitotic competence. This was supported by the finding that the rate of DNA synthesis (indicated by [$^3$H]-thymidine uptake) was decreased, even in light of an augmented component of DNA repair activity (OGG1).

The increased vulnerability of proliferating NSC to OTA was unexpected since DNA repair systems are typically more active and efficient in proliferating cells than in postmitotic differentiated cells (Alexander, 1967; Korr and Schultz, 1989). The accuracy of overall DNA replication during proliferation is assured by several mechanisms that include nucleotide selection and exonuclease proofreading activity with DNA polymerases and postreplicative methylation-instructed mismatch repair system (Krokan *et al.*, 1997). Postmitotic cells contain lower levels of DNA repair enzymes and repair DNA slower than proliferating cells (Alexander, 1967; Korr and Schultz, 1989). Repair of oxo$^6$-alkylguanine lesions as well as the damage from UV and ionizing radiations is slower in postmitotic neurons than in other cultured cell types (Mazzarello *et al.*, 1992). The decline in DNA repair capacity of postmitotic cells may be related to the development and achievement of their terminal differentiated state. Postmitotic cells reduce or ''switch-off'' activity levels of some enzymes and proteins directly or indirectly involved in DNA replication. Aging brain tissue has been reported to have diminished capacity to upregulate DNA repair enzymes when challenged with an oxidative stress such as hyperoxia (Edwards *et al.*, 1998). Not all enzymes involved in neuronal DNA synthesis are switched off and some maintain constant activity levels during the life span of the neuron. For example, DNA polymerase-β is responsible for the whole nuclear DNA polymerase activity in adult neurons.

The clinical significance of the impact of OTA on hippocampal neurogenesis in adult brain relates to its potential effect on cognitive function, provided that effects on neurogenesis observed *in vitro* can be reproduced *in vivo*. It has been suggested that generation of new neurons throughout life is related to the formation of temporal associations in memory (Aimone *et al.*, 2006). Mycotoxin exposure (but not specifically OTA) has been significantly associated with memory loss, learning and attention deficits, processing speed, and debilitated executive functions (Gordon and Cantor, 2004; Gordon *et al.*, 2004; Rea *et al.*, 2003). In addition to cognitive deficits, a significant association between psychological disorders such as melancholic depression with mycotoxin exposure has been reported (Crago *et al.*, 2003). Interestingly, impaired hippocampal neurogenesis may underlie depression, as suggested by the observation that certain antidepressants (selective serotonin uptake inhibitors) stimulate hippocampal neurogenesis (Becker and Wojtowicz, 2007; Huang and Herbert, 2006).

In light of the critical role played by HP in cognitive function, and the importance of neurogenesis in this structure throughout life, the impact of mycotoxins on hippocampal NSC is highly relevant from both molecular pathogenetic and clinical perspectives. The studies reported here are limited by the narrow focus on the activity of a key mammalian base excision repair effector enzyme OGG1 in the OTA-exposed NSC. Modulation of OGG1 was important in determining viability of differentiating NSC, as evidenced by the protective effect of preconditioning with an agent that modulated OGG1 activity. However, a coordinated response to DNA damage requires detection of the damage, transduction of the signal and execution of the repair. Therefore, the activities of other components of the DNA repair response to OTA require further investigation. To determine the potential clinical significance of exposure to OTA, ongoing studies are investigating the impact of subacute and chronic OTA administration on hippocampal-dependent learning paradigms and correlating these cognitive deficits with impaired hippocampal neurogenesis *in vivo*.

## REFERENCES

Abarca, M. L., Bragulat, M. R., Castella, G., and Cabanes, F. J. (1994). Ochratoxin A production by strains of Aspergillus niger var. niger. *Appl. Environ. Microbiol.* **60,** 2650–2652.

Aimone, J. B., Wiles, J., and Gage, F. H. (2006). Potential role for adult neurogenesis in the encoding of time in new memories. *Nat. Neurosci.* **9,** 723–727.

Aleo, M. D., Wyatt, R. D., and Schnellmann, R. G. (1991). Mitochondrial dysfunction is an early event in ochratoxin A but not oosporein toxicity to rat renal proximal tubules. *Toxicol. Appl. Pharmacol.* **107,** 73–80.

Alexander, P. (1967). The role of DNA lesions in processes leading to aging in mice. *Symp. Soc. Exp. Biol.* **21,** 29–50.

Bayman, P., Baker, J. L., Doster, M. A., Michailides, T. J., and Mahoney, N. E. (2002). Ochratoxin production by the Aspergillus ochraceus group and Aspergillus alliaceus. *Appl. Environ. Microbiol.* **68,** 2326–2329.

Becker, S., and Wojtowicz, J. M. (2007). A model of hippocampal neurogenesis in memory and mood disorders. *Trends Cogn. Sci.* **11,** 70–76.

Belmadani, A., Tramu, G., Betbeder, A. M., and Creppy, E. E. (1998). Subchronic effects of ochratoxin A on young adult rat brain and partial prevention by aspartame, a sweetener. *Hum. Exp. Toxicol.* **17,** 380–386.

Byrne, T. N. (2005). Cognitive sequelae of brain tumor treatment. *Curr. Opin. Neurol.* **18,** 662–666.

Campbell, S., and Macqueen, G. (2004). The role of the hippocampus in the pathophysiology of major depression. *J. Psychiatry Neurosci.* **29,** 417–426.

Cardozo-Pelaez, F., Brooks, P. J., Stedeford, T., Song, S., and Sanchez-Ramos, J. (2000). DNA damage, repair, and antioxidant systems in brain regions: A correlative study. *Free Radic. Biol. Med.* **28,** 779–785.

Cardozo-Pelaez, F., Stedeford, T. J., Brooks, P. J., Song, S., and Sanchez-Ramos, J. R. (2002). Effects of diethylmaleate on DNA damage and repair in the mouse brain. *Free Radic. Biol. Med.* **33,** 292–298.

Chen, H., Tung, Y. C., Li, B., Iqbal, K., and Grundke-Iqbal, I. (2006). Trophic factors counteract elevated FGF-2-induced inhibition of adult neurogenesis. *Neurobiol. Aging.*

Crago, B. R., Gray, M. R., Nelson, L. A., Davis, M., Arnold, L., and Thrasher, J. D. (2003). Psychological, neuropsychological, and electrocortical effects of mixed mold exposure. *Arch. Environ. Health* **58,** 452–463.

Creppy, E. E., Kane, A., Dirheimer, G., Lafarge-Frayssinet, C., Mousset, S., and Frayssinet, C. (1985). Genotoxicity of ochratoxin A in mice: DNA single-strand break evaluation in spleen, liver and kidney. *Toxicol. Lett.* **28,** 29–35.

Das, D. K., Maulik, N., Sato, M., and Ray, P. S. (1999). Reactive oxygen species function as second messenger during ischemic preconditioning of heart. *Mol. Cell. Biochem.* **196,** 59–67.

Edwards, M., Rassin, D. K., Izumi, T., Mitra, S., and Perez-Polo, J. R. (1998). APE/Ref-1 responses to oxidative stress in aged rats. *J. Neurosci. Res.* **54,** 635–638.

Elstner, E. F., and Heupel, A. (1976). Inhibition of nitrite formation from hydroxyl-ammonium chloride: A simple assay for superoxide dismutase. *Anal. Biochem.* **70,** 616–620.

Esteban-Pretel, G., and Pilar Lopez-Garcia, M. (2006). An experimental design for the controlled modulation of intracellular GSH levels in cultured hepatocytes. *Free Radic. Biol. Med.* **41,** 610–619.

Fukui, Y., Hayasaka, S., Itoh, M., and Takeuchi, Y. (1992). Development of neurons and synapses in ochratoxin A-induced microcephalic mice: A quantitative assessment of somatosensory cortex. *Neurotoxicol. Teratol.* **14,** 191–196.

Gordon, W. A., and Cantor, J. B. (2004). The diagnosis of cognitive impairment associated with exposure to mold. *Adv. Appl. Microbiol.* **55,** 361–374.

Gordon, W. A., Cantor, J. B., Johanning, E., Charatz, H. J., Ashman, T. A., Breeze, J. L., Haddad, L., and Abramowitz, S. (2004). Cognitive impairment associated with toxigenic fungal exposure: A replication and extension of previous findings. *Appl. Neuropsychol.* **11,** 65–74.

Grote, H. E., Bull, N. D., Howard, M. L., van Dellen, A., Blakemore, C., Bartlett, P. F., and Hannan, A. J. (2005). Cognitive disorders and neurogenesis deficits in Huntington's disease mice are rescued by fluoxetine. *Eur. J. Neurosci.* **22,** 2081–2088.

Hasegawa, E., Takeshige, K., Oishi, T., *et al.* (1990). MPP+ induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochem. Biophys. Res. Commun.* **170,** 1049–1055.

Hayes, A. W., Cain, J. A., and Moore, B. G. (1977). Effect of aflatoxin B1, ochratoxin A and rubratoxin B on infant rats. *Food Cosmet. Toxicol.* **15,** 23–27.

Hayes, A. W., Hood, R. D., and Lee, H. L. (1974). Teratogenic effects of ochratoxin A in mice. *Teratology* **9,** 93–97.

Hong, J. T., Park, K. L., Han, S. Y., Park, K. S., Kim, H. S., Oh, S. D., Lee, R. D., and Jang, S. J. (2000). Effects of ochratoxin A on cytotoxity and cell differentiation in cultured rat embryonic cells. *J. Toxicol. Environ. Health A* **61,** 609–621.

Huang, G. J., and Herbert, J. (2006). Stimulation of neurogenesis in the hippocampus of the adult rat by fluoxetine requires rhythmic change in corticosterone. *Biol. Psychiatry* **59,** 619–624.

Huff, J. E. (1991). Carcinogenicity of ochratoxin A in experimental animals. *IARC Sci. Publ.* 229–244.

Jonsyn-Ellis, F. E. (2000). Seasonal variation in exposure frequency and concentration levels of aflatoxins and ochratoxins in urine samples of boys and girls. *Mycopathologia* **152,** 35–40.

Kawai, T., Takagi, N., Mochizuki, N., Besshoh, S., Sakanishi, K., Nakahara, M., and Takeo, S. (2006). Inhibitor of vascular endothelial growth factor receptor tyrosine kinase attenuates cellular proliferation and differentiation to mature neurons in the hippocampal dentate gyrus after transient forebrain ischemia in the adult rat. *Neuroscience* **141,** 1209–1216.

Korr, H., and Schultz, B. (1989). Unscheduled DNA synthesis in various types of cells of the mouse brain in vivo. *Exp. Brain Res.* 573–578.

Krokan, H. E., Standal, R., and Slupphaug, G. (1997). DNA glycosylases in the base excision repair of DNA. *Biochem. J.* **325,** 1–16.

Kuiper-Goodman, T., and Scott, P. M. (1989). Risk assessment of the myco-toxin ochratoxin A. *Biomed. Environ. Sci.* **2,** 179–248.

Lagace, D. C., Yee, J. K., Bolanos, C. A., and Eisch, A. J. (2006). Juvenile administration of methylphenidate attenuates adult hippocampal neuro-genesis. *Biol. Psychiatry* **60,** 1121–1130.

Marquardt, R. R., and Frohlich, A. A. (1992). A review of recent advances in understanding ochratoxicosis. *J. Anim. Sci.* **70,** 3968–3988.

Mazzarello, P., Poloni, M., Spadari, S., and Focher, F. (1992). DNA repair mechanisms in neurological diseases: Facts and hypotheses. *J. Neurol. Sci.* **112,** 4–14.

Mignone, J. L., Kukekov, V., Chiang, A. S., Steindler, D., and Enikolopov, G. (2004). Neural stem and progenitor cells in nestin-GFP transgenic mice. *J. Comp. Neurol.* **469,** 311–324.

Monje, M. L., Mizumatsu, S., Fike, J. R., and Palmer, T. D. (2002). Irradiation induces neural precursor-cell dysfunction. *Nat. Med.* **8,** 955–962.

Monje, M. L., and Palmer, T. (2003). Radiation injury and neurogenesis. *Curr. Opin. Neurol.* **16,** 129–134.

Murry, C. E., Jennings, R. B., and Reimer, K. A. (1986). Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation* **74,** 1124–1136.

Nacher, J., and McEwen, B. S. (2006). The role of N-methyl-D-asparate receptors in neurogenesis. *Hippocampus* **16,** 267–270.

Palmer, T. D., Takahashi, J., and Gage, F. H. (1997). The adult rat hippo-campus contains primordial neural stem cells. *Mol. Cell. Neurosci.* **8,** 389–404.

Pfohl-Leszkowicz, A., Grosse, Y., Castegnaro, M., Nicolov, I. G., Chernozemsky, I. N., Bartsch, H., Betbeder, A. M., Creppy, E. E., and Dirheimer, G. (1993a). Ochratoxin A-related DNA adducts in urinary tract tumours of Bulgarian subjects. *IARC Sci. Publ.* 141–148.

Pfohl-Leszkowicz, A., Grosse, Y., Kane, A., Creppy, E. E., and Dirheimer, G. (1993b). Differential DNA adduct formation and disappearance in three mouse tissues after treatment with the mycotoxin ochratoxin A. *Mutat. Res.* **289,** 265–273.

Rea, W. J., Didriksen, N., Simon, T. R., Pan, Y., Fenyves, E. J., and Griffiths, B. (2003). Effects of toxic exposure to molds and mycotoxins in building-related illnesses. *Arch. Environ. Health* **58,** 399–405.

Rossi, C., Angelucci, A., Costantin, L., Braschi, C., Mazzantini, M., Babbini, F., Fabbri, M. E., Tessarollo, L., Maffei, L., Berardi, N., *et al.* (2006). Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. *Eur. J. Neurosci.* **24,** 1850–1856.

Sava, V., Reunova, O., Velasquez, A., Harbison, R., and Sanchez-Ramos, J. (2006a). Acute neurotoxic effects of the fungal metabolite ochratoxin-A. *Neurotoxicology* **27,** 82–92.

Sava, V., Reunova, O., Velasquez, A., and Sanchez-Ramos, J. (2006b). Can low level exposure to ochratoxin-A cause parkinsonism? *J. Neurol. Sci.* **249,** 68–75.

Scheffler, B., Walton, N. M., Lin, D. D., Goetz, A. K., Enikolopov, G., Roper, S. N., and Steindler, D. A. (2005). Phenotypic and functional characterization of adult brain neuropoiesis. *Proc. Natl. Acad. Sci. USA* **102,** 9353–9358.

Skyschally, A., Schulz, R., Gres, P., Korth, H. G., and Heusch, G. (2003). Attenuation of ischemic preconditioning in pigs by scavenging of free

oxyradicals with ascorbic acid. *Am. J. Physiol. Heart Circ. Physiol.* **284,** H698–H703.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150,** 76–85.

Song, H. J., Stevens, C. F., and Gage, F. H. (2002). Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. *Nat. Neurosci.* **5,** 438–445.

Stedeford, T., Cardozo-Pelaez, F., Nemeth, N., Song, S., Harbison, R. D., and Sanchez-Ramos, J. (2001). Comparison of base-excision repair capacity in proliferating and differentiated PC 12 cells following acute challenge with dieldrin. *Free Radic. Biol. Med.* **31,** 1272–1278.

Tamaru, M., Hirata, Y., and Matsutani, T. (1988). Neurochemical effects of prenatal treatment with ochratoxin A on fetal and adult mouse brain. *Neurochem. Res.* **13,** 1139–1147.

Wangikar, P. B., Dwivedi, P., Sharma, A. K., and Sinha, N. (2004). Effect in rats of simultaneous prenatal exposure to ochratoxin A and aflatoxin B(1). II. Histopathological features of teratological anomalies induced in fetuses. *Birth Defects Res. B Dev. Reprod. Toxicol.* **71,** 352–358.

Wei, Y. H., Lu, C. Y., Lin, T. N., and Wei, R. D. (1985). Effect of ochratoxin A on rat liver mitochondrial respiration and oxidative phosphorylation. *Toxicology* **36,** 119–130.

Zepnik, H., Pahler, A., Schauer, U., and Dekant, W. (2001). Ochratoxin A-induced tumor formation: Is there a role of reactive ochratoxin A metabolites? *Toxicol. Sci.* **59,** 59–67.